AD NUMBER

AD856552

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AUTHORITY

SMUFD, per d/a ltr dtd 17 Feb 1972

TRANSLATION NO. [redacted] 2446

DATE: [redacted] June 1969

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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Note:- Errors in the translation are underlined thus _____
The corrected versions are given at the end of the article.

ISOLATION OF ANTIBODIES FROM THE ANTIGEN-ANTIBODY COMPLEX BY ULTRASOUND

2446

(Vydeleniye antitel iz kompleksa antigen-antitelo pri pomoshchi ul'trazvuka)

by

I.F. Mikhailov and I.F. Para

Zh. mikrobiol. epidemiol. immun. 1964 (1) pp. 112-119.

The use of purified antibodies with precisely established properties is a necessary methodological condition for solving a number of problems in modern immunology and immuno-chemistry (antibody structure, mechanism and nature of the antigen-antibody reaction, etc). This accounts for the great interest shown in devising methods for isolating antibodies in the pure state.

A large number of methods have been proposed for obtaining pure antibodies to soluble and insoluble antigens based on isolation of antibodies from a specific antigen-antibody complex. Various procedures have been used to dissociate the specific complex: heating at 56° (Landsteiner and Miller, 1925; Jenkins, 1946), effect of high concentrations of salts (Felton, 1932, Heidelberger and Kendall, 1936; Heidelberger and Kabat, 1938; Duncan, 1937), effect of acids and alkalis (Lee and Wu, 1940; Campbell et al 1951; Sutherland and Campbell, 1958; Zil'ber and Abelev, 1962; Olovnikov, 1962; and others).

The methods employed to dissociate the specific complexes sometimes modify the immune proteins which, as is known, are highly sensitive to heat and concentrated solutions of salts, acids and alkalis. The attendant changes are not always reversible. Therefore, it is necessary to look for other methods of dissociating the specific complex.

The main object of the present work was to attempt to use ultrasound to dissociate the antigen-antibody complex, formed as a result of specific adsorption of the immune serum by an homologous microbe, with subsequent isolation of pure antibodies.

We used Shigella paradysenteriae c (standard strain No. 170), Corynebacterium diphtheriae (toxigenic strain No. 738, type IV) and a tularaemia diagnostic antigen and also the corresponding non-adsorbed

immune sera. In one of the experiments we used polyvalent dysentery (Flexner) antiserum labelled with fluorescein isocyanate.

The technique of the main experiments was as follows. A mixture of equal volume (2.5 ml) of two different immune sera was prepared homologous with two species of microbes known to have a different antigenic structure. Such mixtures were prepared from dysentery and tularaemia, or from dysentery and diphtheria antisera. The titre of agglutinins to the two corresponding microbial species was determined in the mixture of antisera.

The antibodies were extracted from the antiserum mixture with one of the two microorganisms of each pair. For this purpose to the mixture was added in equal volume (5 ml), an 18 hr culture of the corresponding bacteria (or tularaemia diagnostic antigen) washed from agar with saline (0.15 M NaCl, pH 7.0) and containing 25×10^9 microbial cells per ml (against the optical standard). The mixture of sera with adsorbent was placed for 2 hr in the thermostat at 37°, then for 18 hr in a refrigerator at 4°. The quantitative proportions of antiserum and specific adsorbent (bacterial cells) were such as to give incomplete depletion of the corresponding antiserum, which in our view should ensure maximum adsorption of antibodies on each microbial cell.

The material obtained was centrifuged for 30 min at 5000 rpm. The antibody titres to both bacterial species used in the experiment were determined in the supernatant. The sediment of bacteria resuspended in saline to the former volume was thrice washed and centrifuged by the same schedule. The antibody titres to the two microbial species were determined in each portion of the supernatant.

After the third washing the sediment of bacteria (agglutinate) was again resuspended in saline to the former volume and exposed to ultrasound. In the experiments we used a RFT generator with an output of 100 W and intensity ~ 3 W/cm^2 with a frequency of 800 kc/s. Insonation was carried out on a water bath for 45 min. The insonated suspension was spun down for an hour at 15,000 rpm, the sediment discarded and the antibody titres to both microorganisms determined in the supernatant.

This experimental arrangement allowed us to solve two problems:

(1) see whether it was possible to dissociate the specific complex by ultrasound with subsequent isolation of the pure antibodies and (2) demonstrate absence of unspecific serum proteins (heterologous antibodies) introduced into the initial mixture.

This technique was employed in eight experiments giving uniform results. Below are given the protocol details for the three variants of the main experiments.

In one of the variants we used a mixture of dysentery and diphtheria antisera (Table 1). After pooling (reciprocal dilution) these antisera the agglutinin titre in relation to Corynebacterium diphtheriae was 5000 and in relation to the Shigella organisms 10,000. A live Flexner dysentery culture was added to the mixed antisera as adsorbent. After adsorption the antibody titres in the mixture fell in relation not only to the adsorbent microbe (3000) but also in relation to the heterologous Corynebacterium diphtheriae (2000). On first washing the adsorbent the supernatant was found to contain antibodies both to diphtheria (500) and dysentery bacteria (100). The supernatants obtained at the second and third washings contained no agglutinins to either organism.

These observations show that unspecific serum proteins (including heterologous antibodies) entered the specific agglutinate as it formed but the resulting linkage was not strong and adsorbed unspecific proteins can be removed fairly easily by subsequent washings.

After ultrasonic treatment of the resuspended adsorbent and subsequent centrifugation, antibodies (3000) were found in the supernatant only in relation to bacteria homologous with the adsorbent (Shigella flexneri) while the eluate did not react with diphtheria organisms.

Similar results were obtained in another experimental variant for isolating pure antibodies in which we studied a mixture of dysentery and tularaemia antisera (Table 2). Adsorption was also by Flexner dysentery bacteria.

In the third experimental variant a mixture of fluorescent polyvalent dysentery antiserum labelled with fluorescein isocyanate and non-fluorescent tularaemia antiserum was adsorbed with tularaemia diagnostic antigen

(Table 3). By the use of fluorescent antiserum the fluorescent analysis for deciding whether unspecific proteins were absent from the eluate was highly sensitive.

The results did not essentially differ from those of the previous experiments. The eluate of pure antibodies obtained did not fluoresce in ultra-violet. Treatment of the dysentery bacteria and tularaemia diagnostic antigen fixed on glass with the eluate did not cause them to glow under the fluorescent microscope.

In another experiment in this series a mixture of the same antisera was adsorbed with dysentery bacillus. In this case the eluate fluoresced in ultrd-violet. On treatment of Flexner bacillus and the tularaemia diagnostic antigen fixed on glass with eluate a faint (+) specific colour of the homologous bacteria and absence of colour for the heterologous forms was seen under the fluorescent microscope.

The specificity of the pure isolated antibodies and also agglutination of homologous bacteria was clearly established in the experiments with staining of the homologous bacteria by the indirect fluorescent antibody method. Fig. 1 indicates the bright (++++) specific fluorescence of Flexner dysentery bacteria treated with the homologous antibodies of the eluate. Specific adsorption of the latter on the bacteria was established by use of sheep antiserum to rabbit labelled with fluorescein isocyanate (indirect fluorescent-antibody method).

Both in this and other similar experiments it was necessary to make sure that the preparations of pure antibodies were free of antigens of the adsorbent which might have passed into solution as a result of partial disintegration of the bacteria by ultrasound. In all the experiments the precipitation test with eluate (antigen) and serum homologous with the adsorbent was negative, while in the controls with bacterial antigen incubated in the same conditions and homologous serum a distinct precipitation ring formed.

Apparently, during extraction of antibodies from the specific complex by ultrasound there is partial disintegration of the bacteria. However, these antigens were precipitated by the extracted antibodies and removed on subsequent centrifugation.

The starting dysentery antiserum and the eluate of pure antibodies obtained from it were studied by paper electrophoresis followed by densitometry of the phoretograms. The eluate was first concentrated three times by blowing in a collodion bag for 5 hr with a table ventilator (Kober method).

Electrophoresis was carried out for 12 hr at 4 V on a paper strip of length 1 cm and current of 0.4 mA for each centimetre of the total width of the phoretograms. Veronal buffer was used at pH 8.6 with ionic strength of 0.11. The results of electrophoresis are given in Fig. 2.

Four protein fractions were detected in the initial immune serum (B) with a relative content of albumin 39.7%, α-globulin 11.4%, β-globulin 21.8%, and γ-globulin 27.1%. No traces of albumin were found in eluate (A).

The antibodies were represented by α-globulin (50%) but β-globulins (21.5%) and γ-globulins (28.5%) were also present. It was thus established that the antibodies isolated from the complex were associated in the eluate not only with γ-globulin but with other globulin fractions. This corresponds to the quite considerable volume of information published on the subject. Konikov (1948) for example, notes presence of antibodies both in the β- and intermediate (between β and γ) fractions detected in immune sera and designated as $_2$ globulin and the T component. According to Rojnek, Rybak and Skavril the O and H antibodies of typhoid antiserum are found in a mixture of α and β-globulins.

The facts presented show that the technique tested by us for dissociatin a complex of particulate antigen plus antibodies by means of ultrasound yielded pure antibodies eluting directly into saline. The special experimental arrangement enabled us to establish absence in the eluate of heterologous antibodies introduced into the initial mixture of test and heterologous antisera.

The specificity of the pure antibodies obtained was confirmed in agglutination tests and also by use of direct and indirect fluorescent-antibody methods. Electrophoretic study of the eluate showed it to be free of non-immune serum proteins. Comparison of the agglutination titres

of the initial antiserum after adsorption and the eluates indicated a yield of pure antibodies of 15-20 per cent.

It would be desirable as a next step to apply more intense ultrasonic treatment to raise the antibody yield.

Summary

1. Using ultrasound we have eluted into saline antibodies from a specific complex of antibodies and particulate antigen.

2. The antibody preparations obtained were free of unspecific serum proteins and heterologous antibodies and also antigens of the immune adsorbent.

3. The antibodies isolated retained their immunological specificity.

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Table 1. Elution of antibodies with ultrasound from bacterial adsorbent using a mixture of diphtheria and dysentery (Flexner) antisera

Purpose of experiment	Reagents: Antibody	Reagents: Antigen (culture)	Result of agglutination test with different dilutions of serum: 1:100	1:500	1:1 000	1:2 000	1:3000	1:5 000	1:10 000	1:15 000	Serum control
Determination of titre of initial sera	Diphtheria antiserum	Diphtheria	+++	++	++	++	++	+	+	-	-
	Dysentery antiserum	Dysentery	++++	++++	++++	+++	+++	++	++	+	-
Determination of antibody titres after pooling of sera 1:1	Mixture of unadsorbed diphtheria and dysentery antisera	Diphtheria	++	++	+	+	+	+	-	-	-
		Dysentery	++++	++++	++++	+++	+++	++	+	-	-
Determination of antibody titres in mixture of sera after adsorbing it with a Flexner culture	Mixture of sera after adsorption with dysentery culture	Diphtheria	++	++	++	+	±	-	-	-	-
		Dysentery	+++	+++	+++	++	+	±	-	-	-
Determination of antibody titre in saline after washing adsorbent	Washing No. 1	Diphtheria	++	+	-	-	-	-	-	-	-
		Dysentery	++	-	-	-	-	-	-	-	-
	Washing Nos 2 and 3	Diphtheria	-	-	-	-	-	-	-	-	-
		Dysentery	-	-	-	-	-	-	-	-	-
*Determination of antibody titre in saline after washing adsorbent and separating antigen by centrifugation	Saline containing individual antibodies	Diphtheria	-	-	-	-	-	-	-	-	-
		Dysentery	+++	++	++	+	±	-	-	-	-

Notes. - (Also in tables 2 and 3) ++++, +++, ++, +, ± standard ratings for positive tests, - negative result.

* In the original Russian text no reference is made here to ultrasonic treatment.

Table 2. Elution of antibodies with ultrasound from Flexner bacteria adsorbent with use of a mixture of tularaemia and dysentery antisera

Purpose of experiment	Reagents: Antibody	Reagents: Antigen (culture)	Result of agglutination test with different dilutions of sera: 1:100	1:500	1:1 000	1:2 000	1:3 000	1:5 000	1:10 000	1:15 000	Serum control
Determination of titres of initial antisera	Dysentery antiserum	Dysentery culture	++++	++++	++++	++++	++++	++++	++	+	-
	Tularaemia antiserum	Tularaemia diagnostic antigen	++++	++++	++++	++++	+++	++	-	-	-
Determination of antibody titre after pooling sera 1:1	Mixture of unadsorbed dysentery and tularaemia antisera (1:1)	Dysentery culture	++++	++++	++++	++++	++++	++++	++	-	-
		Tularaemia diagnostic antigen	++++	++++	++++	++++	++++	++	-	-	-
Determination of antibody titres in antiserum mixture after adsorbing it with Flexner culture	Serum mixture after adsorbing it with dysentery culture	Dysentery culture	++++	++++	++++	+++	++	+	+	-	-
		Tularaemia diagnostic antigen	++++	++++	++++	++++	++++	++	-	-	-
Determination of antibody titre in saline after washing adsorbent	Washing No. 1	Dysentery culture	++	+	-	-	-	-	-	-	-
		Tularaemia diagnostic antigen	+++	++	+	-	-	-	-	-	-
	Washing Nos 2 and 3	Dysentery culture	-	-	-	-	-	-	-	-	-
		Tularaemia diagnostic antigen	-	-	-	-	-	-	-	-	-
Determination of antibody titre in saline after incubating suspension of adsorbent and separating antigen by centrifugation	Saline containing individual antibodies	Dysentery culture	+++	++	++	+	-	-	-	-	-
		Tularaemia diagnostic antigen	-	-	-	-	-	-	-	-	-

Table 3. Elution of antibodies by ultrasound from tularaemia diagnostic antigen (adsorbent) using mixture of tularaemia and fluorescent polyvalent dysentery antiserum

Purpose of Experiment	Reagents		Result of agglutination test with different dilutions of serum								
	Antibody	Antigen	1:100	1:500	1:1 000	1:2 000	1:3 000	1:5 000	1:10 000	1:15 000	Serum control
Determination of titres of initial antisera	Polyvalent (fluorescent) dysentery antiserum	Dysentery culture	++++	++++	++++	+++	-	-	-	-	-
	Tularaemia antiserum	Tularaemia diagnostic antigen	++++	+++	++	+	-	-	-	-	-
Determination of titres after pooling antisera 1 : 1	Mixture of unadsorbed dysentery and tularaemia antisera 1 : 1	Dysentery culture	+++	++	+	-	-	-	-	-	-
		Tularaemia diagnostic antigen	+++	+++	++	+	±	-	-	-	-
Determination of antibody titre in antiserum mixture after adsorbing it with tularaemia diagnostic antigen	Mixture of sera after adsorbing with tularaemia diagnostic antigen	Dysentery culture	+++	++	+	-	-	-	-	-	-
		Tularaemia diagnostic antigen	++	+	-	-	-	-	-	-	-
Determination of antibody titre in saline after washing absorbent	Washing No. 1	Dysentery culture	+	-	-	-	-	-	-	-	-
		Tularaemia diagnostic antigen	-	-	-	-	-	-	-	-	-
	Washing Nos. 2 and 3	Dysentery culture	-	-	-	-	-	-	-	-	-
		Tularaemia diagnostic antigen	-	-	-	-	-	-	-	-	-
Determination of antibody titre in saline after insonating suspension of adsorbent and separating antigen by centrifugation	Saline containing individual antibodies	Dysentery culture	-	-	-	-	-	-	-	-	-
		Tularaemia diagnostic antigen	++	±	-	-	-	-	-	-	-

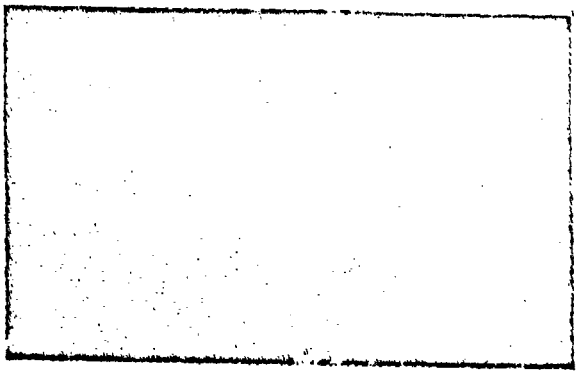
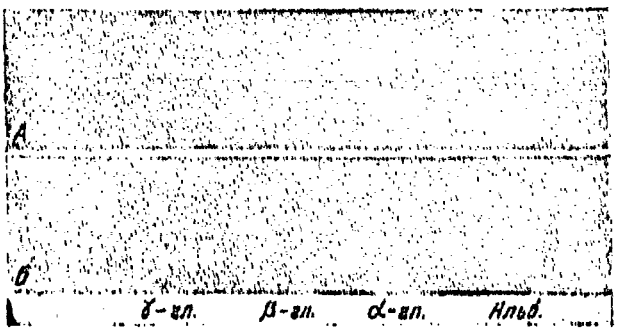


Fig. 1. Specific fluorescence of Shigella flexneri 6 (indirect fluorescent-antibody method)

Photo. Mi-1, ob. 90x, oc. 7x.



γ-globulin β-globulin α-globulin Albumin

Fig. 2. Electrophoretogram of eluate of specific antibodies (A) isolated from dysentery (Flexner) antiserum (B) by exposing homologous bacterial adsorbent to ultrasound.

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CORRECTIONS

Page	Line	Comment
1	1	for "THE ANTIGEN-ANTIBODY" read "ANTIGEN-ANTIBODY"
1	2	for "COMPLEX" read "COMPLEXES"
1	24	for "immune proteins" read "immunoglobulins"
1	30	for "absorption of the immune serum by homologous microby" read "adsorption of antisera by its 'homologoud organism'"
2	1	for "immune sera" read "antisera"
2	4,5,6	for "of equal volume (2.5ml) of two different immune sera was prepared homologous with two species of microbes known to have a different antigenic structure." read "was prepared of equal volumes (2.5ml) of two specific antisera against two species of organisms known to have different antigenic structure"
2	7	for "titre of agglutinins" read "agglutinins titre"
2	8	for "microbial species" read "organisms"
2	9	for "extracted" read "adsorbed"
2	24	for "mi[illegible]bial" read "bacterial"
2	27	for "exposed to ultrasound" read "treated ultrasonically"
2	29	for "Insonation" read "Ultrasonic treatment"
2	30	for "insonated" read "treated"
3	10	for "agglutinin" read "agglutination"
4	29	for "insonated in" read "treated ultrasonically under"
5	3,4,5	for "The eluate was first concentrated three times by blowing in a collodion bag for 5 hr with a table ventilator (Kober method)." read "The eluate was first concentrated three-fold by evaporation for 5 hr in a collodion bag using a table fan (Kober method)."

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